November 2000

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 275, No. 45, Issue of November 10, pp. 35540–35547, 2000
Printed in U.S.A.

Structure-Expression Relationships of the 15-kDa Selenoprotein Gene

POSSIBLE ROLE OF THE PROTEIN IN CANCER ETIOLOGY*

Received for publication, May 10, 2000, and in revised form, July 24, 2000
Published, JBC Papers in Press, August 16, 2000, DOI 10.1074/jbc.M004014200

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Selenium has been implicated in cancer prevention, but the mechanism and possible involvement of selenoproteins in this process are not understood. To elucidate whether the 15-kDa selenoprotein may play a role in cancer etiology, the complete sequence of the human 15-kDa protein gene was determined, and various characteristics associated with expression of the protein were examined in normal and malignant cells and tissues. The 51-kilobase pair gene for the 15-kDa selenoprotein consisted of five exons and four introns and was localized on chromosome 1p31, a genetic locus commonly mutated or deleted in human cancers. Two stem-loop structures resembling selenocysteine insertion sequence elements were identified in the 3′-untranslated region of the gene, and only one of these was functional. Two alleles in the human 15-kDa protein gene were identified that differed by two single nucleotide polymorphic sites that occurred within the selenocysteine insertion sequence-like structures. These 3′-untranslated region polymorphisms resulted in changes in selenocysteine incorporation into protein and responded differently to selenium supplementation. Human and mouse 15-kDa selenoprotein genes manifested the highest level of expression in prostate, liver, kidney, testis, and brain, and the level of the selenoprotein was reduced substantially in a malignant prostate cell line and in hepatocarcinoma. The expression pattern of the 15-kDa protein in normal and malignant tissues, the occurrence of polymorphisms associated with protein expression, the role of selenium in differential regulation of polymorphisms, and the chromosomal location of the gene may be relevant to a role of this protein in cancer.

The essential trace element selenium occurs in several proteins found in bacteria (1), archaea (2), and eukaryotes (1, 3). All of the known selenoproteins described to date incorporate selenium into protein co-translationally as the amino acid selenocysteine (Sec)[1] in response to the UGA codon (1, 3, 4), with the exception of several bacterial molybdenum-containing enzymes such as *Clostridium barkeri* nicotinic acid hydroxylase that contains an active center dissociable selenium species (5–7). Among the selenoproteins thus far identified in mammals are four glutathione peroxidases, three thioredoxin reductases, three thyroid hormone deiodinases, selenophosphate synthetase, selenoprotein P, selenoprotein W, selenoprotein T, selenoprotein R (also called selenoprotein X), selenoprotein N, and a 15-kDa selenoprotein (1, 3, 8–10).

The 15-kDa selenoprotein was initially identified as a strongly labeled protein that was detected when human T cells were grown in the presence of [^{75}Se]selenite (11). The open reading frame within the human 15-kDa protein cDNA coded for 162 residues and contained an in-frame TGA codon that would result in the incorporation of Sec at codon position 93. A putative stem-loop structure, designated the Sec insertion sequence (SECIS) element, was predicted in the 3′-untranslated region (3′-UTR). SECIS elements are required for insertion of Sec into protein at in-frame TGA codons and are located in the 3′-UTR of higher eukaryotic selenoprotein mRNAs and immediately downstream of the UGA Sec codon in bacterial selenoprotein mRNAs (12). The 15-kDa protein did not have homology to previously characterized proteins, but homologous sequences were detected in other mammals as well as in the nematodes *Caenorhabditis elegans* and *Brugia malayi* (11).

Nutritional levels of selenium have been inversely correlated to the incidence of both prostate and lung cancer in human epidemiological studies (13, 14). In addition, recent supplementation trials suggest that selenium may be effective in the reduction of common human cancers, including those of the prostate, colon and lung (15). Given the presence of selenium in human proteins (1, 3), the absolute developmental requirement for selenoprotein synthesis (16), and that selenium availability at nutritional levels can influence selenoprotein levels (17), it is probable that the chemopreventive properties of this element are mediated through its role in one or more selenoproteins. However, none of the identified selenoproteins have conclu-

* This work was supported by a grant from the Cancer Research Foundation of America (to V. N. G) and in part by a grant from the American Institute for Cancer Research (to A. M. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AF288992 (human 15-kDa protein gene), AF288991 (extended human 15-kDa protein cDNA), and AF288740 (mouse 15-kDa protein cDNA).

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[1] The abbreviations used are: Sec, selenocysteine; SECIS, Sec insertion sequence; TGF, transforming growth factor; kb, kilobase pair; PCR, polymerase chain reaction; UTR, untranslated region; EST, expressed sequence tag.

sively been linked to either the prevention or susceptibility to any human cancers. As a first step in evaluating the biological role of the newly discovered 15-kDa selenoprotein and its possible involvement as a chemopreventive agent in cancer, we have characterized the 15-kDa protein gene in humans and the corresponding cDNA sequence in mice. This analysis revealed a polymorphism in the human SECIS element that influences the level of 15-kDa protein synthesis. In addition, the 15-kDa protein was found to be expressed in high levels in organs that are protected from malignancy by selenium and in reduced levels in the corresponding cancerous organs. These studies, along with the observation that the 15-kDa protein gene maps at a chromosomal location often associated with malignancy (18, 19), raise the possibility that selenium may protect against cancer, at least in part, through its influence on this protein.

EXPERIMENTAL PROCEDURES

Materials—Human liver mRNA was obtained from Invitrogen, mouse cDNA clones from Genome Systems, and total RNA from human tissues (human brain, pooled from two male Caucasians, ages 43 and 47; human heart, pooled from eight male/female Caucasians, ages 25–73; human kidney, pooled from eight male/female Caucasians, ages 25–55; human liver, pooled from one male and one female Caucasian, ages 35 and 15; human lung, pooled from five male/female Caucasians, ages 14–40; human trachea, pooled from 84 male/female Caucasians, ages 19–65; human mammary gland, pooled from eight female Caucasians, ages 16–45; human prostate, pooled from 23 male Caucasians, ages 23–64; human skeletal muscle, pooled from 10 male/female Caucasians, ages 23–56 (muscle of thigh, ileopsoas, and pectoralis major); human testis, pooled from 19 male Caucasians, ages 17–61; and human uterus, pooled from 10 female Caucasians, ages 15–77) and mouse multiple tissue mRNA blots from CLONTECH. The mouse prostate adenocarcinoma cell line Pr-14 (20) was kindly provided by Cheryl Jorcyk and Jeffrey Green (National Institutes of Health). Normal and malignant livers from transforming growth factor α (TGFα)/c-*myc* transgenic mice (21) were obtained as described previously (22). Immunoblot reagents SuperSignal and ECL were obtained from Pierce and Amersham Pharmacia Biotech, respectively, pET-21b(+) vector from Novagen, the QuickChange site-directed mutagenesis kit from Stratagene, Lipofectin Reagent from Life Technologies, Inc., the Ni^{2+}-nitrilotriacetic acid column from Qiagen, the TSK-gel Phenyl 5-PW column from TosoHaas, and the Mono-Q column from Amersham Pharmacia Biotech. Avian myeloblastosis virus reverse transcriptase, Reporter Lysis Buffer, and luciferase and β-galactosidase activity assay systems were obtained from Promega. ThermoFidelase I was from Fidelity Systems. All other reagents were of the highest grade available.

Human Genomic DNA Sequencing—The human cDNA for the 15-kDa protein (11) was used as a probe to search, in a hybridization screen, for a genomic DNA clone in human BAC and PAC libraries from Genome Systems. One ~100-kb clone hybridized with the probe, and the 15-kDa protein gene present in the clone was sequenced using a primer walking strategy. Sequencing was achieved without subcloning using a recently developed procedure for direct sequencing of BAC templates employing ThermoFidelase I (23). The addition of ThermoFidelase I into the cycle sequencing reaction allowed reading through G/C-rich regions and prevented the stuttering of DNA polymerase on single nucleotide repeats. A contiguous 64-kb genomic DNA sequence was generated that contained the 51-kb 15-kDa selenoprotein gene, except that approximately half of intron 2 was taken from a partial sequence of a BAC clone generated by the Human Genome Project (accession number HSDJ604K5).

Transcription Start Site—Primer extension analysis to map the transcription start site of the 15-kDa protein gene was performed according to a previously developed procedure (24). A synthetic oligonucleotide primer (5′-GCCAACAACAACCGTAGC-3′) complementary to the 5′-end of the open reading frame (+96/+79 from the initiation codon) of the selenoprotein gene was 5′-labeled with [α-^{32}P]ATP and hybridized to 0.5 μg of human liver mRNA. The resulting complex was then reverse transcribed using avian myeloblastosis virus reverse transcriptase. Extension products were electrophoresed on an 8% denaturing polyacrylamide gel and visualized by a PhosphorImager.

Mouse cDNA Sequence—Sequences of two mouse cDNA clones (accession numbers for EST clones are AA69039 and W71333) homologous to the human 15-kDa protein sequence were determined using a Dye Terminator Cycle Sequencing kit from PerkinElmer Life Sciences.

Northern and Southern Blot Analyses—Northern blot analyses using the mouse cDNA probe for detecting the 15-kDa protein mRNA were performed on a membrane from CLONTECH. Northern blot analyses to detect the human 15-kDa protein mRNA were performed by running total human RNA on a formaldehyde agarose gel, transferring it onto a Nylon membrane, and hybridizing with a human cDNA as a probe. Southern blot analyses using the human 15-kDa protein cDNA as probe, and hybridization assays were performed as described (24). A blot containing mRNA isolated from mouse testis following sucrose gradient fractionation of polysomal and nonpolysomal RNA and subsequent isolation of RNA from each fraction was prepared as described (25).

Immunoblot Analyses—Rabbit polyclonal antibodies that were raised against the C-terminal peptide in the human 15-kDa protein (11) were used for detection of the selenoprotein in mouse protein extracts. Sequencing of the mouse cDNA revealed that mouse and human selenoproteins had identical C-terminal amino acid sequences. SuperSignal and ECL systems were used for signal detection. The isolated human 15-kDa protein was obtained as described (11) and used as a control for immunoblot assays. The mouse prostate adenocarcinoma cell line Pr-14 was grown as described previously (20). Extracts of malignant and matching normal livers from male TGFα/c-*myc* double-transgenic mice were prepared as described (22) from either the same or different animals as indicated. These mice, generated in a CD1xB6CBA F1 background, overexpress TGFα and oncogene c-*myc*, and represent a well characterized *in vivo* model of accelerated hepatocarcinogenesis (21). Male TGFα/c-*myc* mice develop easily identifiable liver tumors at the age of 6–10 months.

Generation of Constructs for Functional Analysis of a SECIS Element in the Human 15-kDa Selenoprotein Gene—Oligonucleotide primers 5′-CCAAGCTTTAGGCGTTAATGAAGACTACACAG-3′ and 5′-CCGCGG-CCGCTGTATGTATCTGATCCACACAA-3′ complementary to cDNA positions 1092–1112 and 1130–1150 of human 15-kDa selenoprotein were designed with terminal *Hin*dIII and *Not*I sites, respectively. Primers were used to PCR-amplify the minimal SECIS element from the C^{811}/G^{1125} cDNA. Mutagenesis to generate the polymorphic variant element that has A^{1125} in place of G^{1125} was carried out by amplification of an internal mutagenic oligonucleotide 5′-TTAATGAAGA CTACAC-AGAA AACCTTTCTA AGGATTTGTG TGGATCAGATACA-3′ containing the desired nucleotide changes in combination with the above wild-type oligonucleotides encoding the terminal restriction sites. PCR products were digested with the appropriate enzymes and subcloned into the corresponding sites of G16-D10ΔH3 (26). Once subcloned, the amplified regions were sequenced in their entirety.

Transient Transfections—Transient transfections were carried out in human embryonic kidney (HEK 293) cells using the calcium phosphate method of transfection as described previously (27). Three days prior to transfection, HEK 293 cells were plated onto 60-mm culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were transfected with 10 μg of the pUHD10-3-based expression plasmids and 4 μg of the pUHD15 plasmid (28), which encodes a protein necessary for transcriptional activation of the pUHD10-3 promoter. To monitor transfection efficiencies, cells were co-transfected with 3 μg of an expression vector containing the human growth hormone cDNA under control of the hamster sarcoma virus thymidine kinase promoter. Medium was changed 1 day following transfection. Two days after transfection, cells were harvested, washed, and resuspended in 0.1 M potassium phosphate, pH 6.9, containing 1 mM EDTA and 0.25 M sucrose.

Deiodinase Assays—Cells were harvested 48 h after transfection by scraping and sonicated, and cell sonicates were assayed for 5′-deiodinase activity as described previously (27). Briefly, cells were harvested, washed, and resuspended in 0.1 M potassium phosphate, pH 6.9, containing 1 mM EDTA, 0.25 M sucrose, and 10 mM dithiothreitol. Cells were then sonicated and assayed for the ability to 5′-deiodinate ^{125}I reverse T3. Reactions contained 10–250 μg of protein, 1 μM ^{125}I reverse T3, and 10 mM dithiothreitol in a reaction volume of 300 μl. Reactions were incubated at 37 °C for 30 min. ^{125}I release was quantitated as described previously (27). Deiodinase activities were calculated per microliter of cell sonicate and normalized to the amount of growth hormone secreted into the medium. All constructs were tested in at least three separate transfections, and deiodinase assays were performed in duplicate from each transfection.

Reporter Constructs and Analysis—Reporter constructs designed to evaluate SECIS element function were generated using human DNAs for which the nucleotide positions at 811 and 1125 of the 15-kDa 3′-UTR were determined by PCR amplification and diagnostic restriction en-

zyme digestion.[2] A 533-nucleotide PCR amplification product containing the SECIS element was generated using human DNA as a template and primers including the restriction endonuclease recognition sequence for either *Spe*I (5′-AAAACTAGTGCTTTGTAACAGACTTGCGGTTAATTATGC-3′) or *Pst*I (5′-AAACTGCAGGGTCTTACAAATGATCACTTTTAAATGGAC-3′). PCR products were cleaved with those enzymes and directionally inserted into the polycloning site of the pBPLUGA vector (30). The reporter construct was co-transfected into mouse fibroblasts (NIH 3T3) with the pSV_2neo plasmid at a 10:1 molar ratio using the Lipofectin reagent. Lysates were generated by a single freeze-thaw cycle following suspension in Reporter Lysis Buffer, and luciferase and β-galactosidase activities were determined using the corresponding assay systems. Luciferase activity was quantified using a Fentomaster FB12 Luminometer from Zylux. For selenium supplementation conditions, the cells were incubated at the indicated concentration of sodium selenite for 5 days prior to lysis.

Computer Search Analyses—Protein and DNA homology analyses were performed with BLAST programs (31). Putative structures for human and mouse 15-kDa protein mRNAs were predicted with mfold (32) and SECISearch (9) programs. The presence of N-terminal signal peptides was determined with sequence analysis programs SignalP and PSORT II.

RESULTS

Sequence and Structural Organization of the Human 15-kDa Protein Gene—A human BAC library was screened with a radioactive probe representing the human 15-kDa protein cDNA resulting in the isolation of a positively hybridizing clone. Restriction enzyme analysis of this clone by Southern blotting indicated that a variety of infrequent cutting restriction endonucleases used separately to digest the BAC insert resulted in several 15-kDa protein cDNA-hybridizing fragments (data not shown). This suggested that the 15-kDa selenoprotein gene was relatively large and constituted a significant portion of the BAC clone. A recently developed technique of direct sequencing of large genomic DNA clones using a ThermoFidelase system was then utilized to determine the insert sequence using primers designed from the selenoprotein cDNA sequence followed by a primer walking strategy to determine intron sequences.

The structural organization of the human 15-kDa protein gene is shown in Fig. 1 and Table I. By comparison of the obtained gene sequence and the previously reported cDNA sequence, the gene for the 15-kDa protein was established to be 51 kb long and consisted of five exons and four introns (Table I). All exon-intron junctions contained the consensus AG/GT sequences that typically flank splice junctions (Table I). Computer search analysis of the determined gene sequence against dbESTs identified several positional markers supporting the previous determination that the gene for the 15-kDa protein is located on chromosome 1 at position 1p31 (data not shown).

The first exon in the selenoprotein gene included the 5′-UTR as well as the first 27 amino acid residues corresponding to a putative signal peptide. This N-terminal peptide, while predicted from the gene sequence, was absent in the isolated human T-cell 15-kDa selenoprotein as evidenced by direct determination of the mass of the isolated selenoprotein and by direct sequencing of the tryptic peptides derived from the 15-kDa protein (11). The coding of an N-terminal signal peptide by a unique exon is often seen for other mammalian proteins. The TGA codon that encodes Sec was located in exon 3, while the largest exon, exon 5, contained the C-terminal coding region and the complete 3′-UTR.

In addition, we determined 6.8 kb in the 5′-flanking and 6.0 kb 3′-flanking regions for the human 15-kDa protein gene. The first 477 nucleotides of the gene for heparan sulfate 2-sulfotransferase (accession number AB007917) were detected in the reverse orientation upstream of the selenoprotein gene within nucleotides 5741–6217 of the BAC clone. The downstream neighbor of the 15-kDa selenoprotein gene could not be identified.

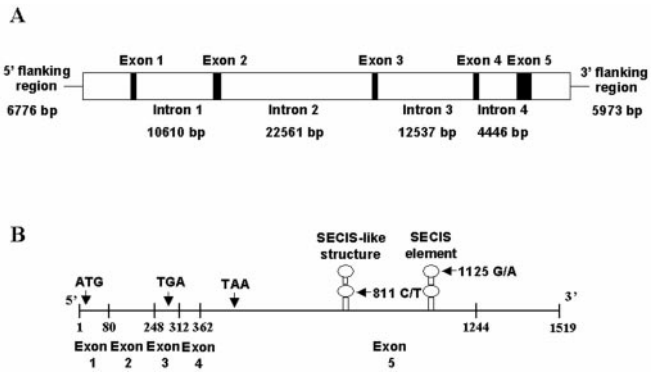

FIG. 1. **Structural organization of the human 15-kDa selenoprotein gene.** *A*, the exon-intron organization and the size of exons and introns in the selenoprotein gene predicted by sequence analysis are shown. *Open squares* correspond to introns and flanking regions, and the *closed squares* correspond to exons. The sizes of introns and flanking regions are indicated. *B*, the organization of the human cDNA sequence and the relative positions of the ATG initiation and the TGA Sec codons, the TAA termination signal, and the detected polymorphisms (C^{811}/T^{811} in the SECIS-like structure and G^{1125}/A^{1125} in the SECIS element) are shown. Alternative 3′-end sequences (position 1244 or 1519 within the cDNA) of exon 5 are also indicated. The *long horizontal line* corresponds to the 15-kDa protein cDNA, and *short vertical lines* correspond to exon-exon junctions. *Numbers under* junction sites correspond to the last nucleotides in the preceding exons. *bp*, base pairs.

A computer search analyses for sequences homologous to the human 15-kDa protein detected a previously deposited sequence on human chromosome 7q31–3q32 (82% identity to the 15-kDa protein cDNA sequence in a 125-nucleotide overlap; accession number AC004925). This sequence lacked introns and was characterized by in-frame stop signals, suggesting that it represented a 15-kDa protein pseudogene that probably arose by reintegration of a cDNA copy of the 15-kDa protein mRNA.

In order to establish the site of transcription initiation, primer extension of an oligonucleotide that complemented the region encoding the N-terminal portion of the coding region (+96/+79 from the initiation codon) was utilized. The primer was extended up to 20 bases beyond the point corresponding to the bases of the open reading frame (Fig. 2). The nucleotide at the initiation site was adenylic acid. The gene did not contain a canonical TATA box consensus sequence about 30 base pairs upstream of the transcription initiation site as is common among many polymerase II transcribed genes. The sequence at the initiation site, AGAAACC (where A designates the transcription start point) is similar to the approximate consensus sequence reported for mammalian polymerase II-transcribed genes that initiate transcription with or without an upstream TATA box (33). The approximate consensus sequence was determined to be $YYA^{+1}N(T/A)YY$. Clearly, the nucleotide at the transcription start point for the 15-kDa protein gene and those nucleotides downstream fit the consensus sequence, while the two purines upstream do not conform.

Analyses of Human EST Sequences—The human 15-kDa protein cDNA was represented in dbEST by more than 200 independent EST sequences. Based on these available sequence data, it was apparent that exon 4, predicted to consist of 50 nucleotides, was missing in ~7.5% of the ESTs (3 out of 42). A transcript lacking exon 4 would be slightly shorter (149 residues in length) and would presumably be a nonfunctional polypeptide. Interestingly, if this alternative splicing was to

[2] Y. J. Hu, K. V. Korotkov, R. Mehta, D. L. Hatfield, C. Rotimi, A. Luke, T. E. Prewitt, R. S. Cooper, W. Stock, E. E. Vokes, M. E. Dolan, V. N. Gladyshev, and A. M. Diamond, manuscript in preparation.

TABLE I
Organization of the 15-kDa protein gene

Exon (size, bp)	Coding information	Intron (size, bp)	Splice donor	Splice acceptor
1 (97)	5′-UTR + coding region for signal peptide	1 (10,610)	AAGCGgtgag	ttcagGTGTC
2 (168)	Coding region	2 (22,561)	AAAAGgtact	tgtagCTGTA
3 (64)	Coding region (selenocysteine)	3 (12,537)	CCAAGgtata	ataagCTTTT
4 (50)	Coding region	4 (4446)	TCAAGgtaag	tgtagTATGT
5 (>883)	Coding region + 3′-UTR			

Exon sequences are shown in uppercase and intron sequences in lowercase. bp, base pairs.

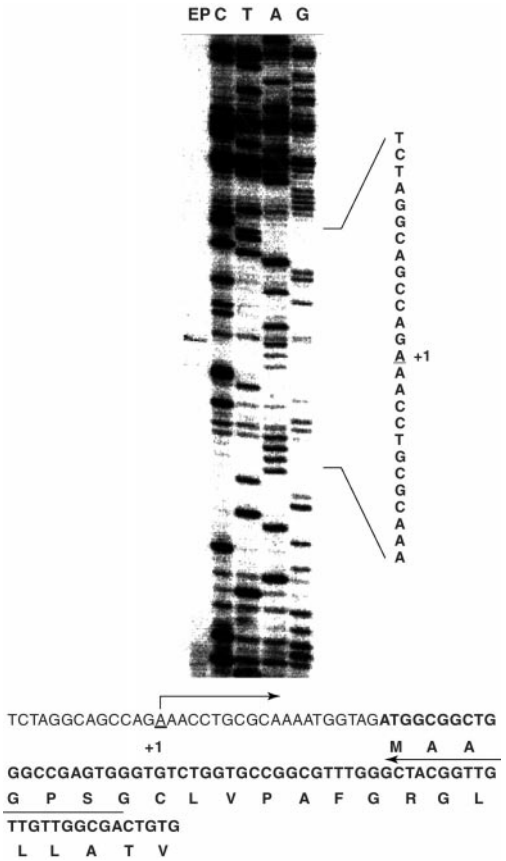

FIG. 2. **Identification of the transcription start site by primer extension analysis.** The analysis was carried out using a ^{32}P-labeled synthetic oligonucleotide primer as described under "Experimental Procedures." Primer extension product is marked as *EP*. *Underlined A* indicates the transcription start site, and the *arrow pointing* to the *right* indicates the direction of transcription. Coding sequences are shown in *boldface type*, and the primer is indicated by the *arrow pointing* to the *left*.

occur in the human 15-kDa protein gene, two in-frame TGA codons downstream of the TGA Sec codon would be present. Translation of this transcript would yield a protein containing three Sec residues. Determining whether such a polypeptide is indeed expressed in human cells will require further studies. However, in preliminary experiments we could not detect DNA sequences lacking exon 4 when total human brain, kidney, liver, heart, and lung RNAs were amplified by reverse transcriptase-PCR with primers corresponding to exons 3 and 5, followed by sequencing of PCR products. In contrast, the sequence containing exon 4 was observed by this method (data not shown).

The presence of a SECIS element in the 3′-UTR of eukaryotic selenoprotein genes is necessary and sufficient to decode in-frame TGA codons as Sec (12). The human 15-kDa protein gene has a single functional SECIS element (see below). Unlike this and other known mammalian selenoprotein genes that encode a single Sec residue and contain a single SECIS element, the selenoprotein P gene encodes 10–12 Sec residues and contains two distinct SECIS elements (34). It is not known if a single SECIS element in a mammalian selenoprotein gene is sufficient to incorporate multiple Sec residues, such as those that may appear in the 15-kDa selenoprotein derived from an alternative transcript lacking the fourth exon.

Two polymorphic sites (nucleotide positions 811 and 1125 in the human cDNA) located in the 3′-UTR were previously predicted in the 15-kDa protein gene from an analysis of the EST data base (11). We have expanded on this initial analysis to include currently available human EST sequences. The analysis indicated that C^{811} nucleotide was associated with G^{1125}, while T^{811} was associated with A^{1125} in every EST sequence that contained both polymorphic sites, suggesting the presence of only two alleles, C^{811}/G^{1125} and T^{811}/A^{1125}. We found that at position 811 in the cDNA sequence, T occurred 19 times, while C was present in 32 ESTs. Likewise, A at position 1125 occurred in 37 sequences, while G occurred in 89 sequences at this position. This transforms into ~32%/68% frequency distribution of T^{811}/A^{1125} and C^{811}/G^{1125} alleles in the submitted sequences. No sex, tissue, or age differences in distribution were found between the two polymorphic forms of the gene based on the EST analysis.

It was also observed that 20 out of 141 EST sequences that corresponded to the 3′-end of the human 15-kDa protein gene were extended beyond previously reported sequences for an additional 275 nucleotides. Examination of extended sequences revealed that they originated from a direct continuation of exon 5 due to an alternative distal atypical poly(A) site usage. The extended sequences showed no homology to previously characterized sequences and contained no SECIS-like structures. The complete cDNA sequence corresponding to the longer transcript and containing C^{811}/G^{1125} polymorphisms has been determined by sequencing an EST clone (accession number AA001402).

SECIS Element Functional Studies—In the human 15-kDa protein cDNA sequence, the G^{1125}/A^{1125} polymorphic site is located in the apical loop of the putative SECIS element (Fig. 3). To determine if this stem-loop structure was indeed involved in Sec incorporation, we utilized a thyroid hormone deiodinase system previously used extensively to determine the efficiency of wild type and mutant SECIS elements in mammalian selenoproteins (27). A construct was made in which the putative SECIS element from the 15-kDa selenoprotein gene replaced the SECIS element in the deiodinase gene. As a positive control for SECIS function, the deiodinase gene containing the wild type SECIS element was used. Transfection of these constructs into human embryonic kidney 293 cells showed that the SECIS element in the 15-kDa protein gene was functional and that its efficiency of Sec incorporation was approximately one-half of that of the deiodinase natural SECIS element (Fig. 4). However, replacement of G^{1125} with an A in the apical loop of the SECIS element increased incorporation of Sec into deio-

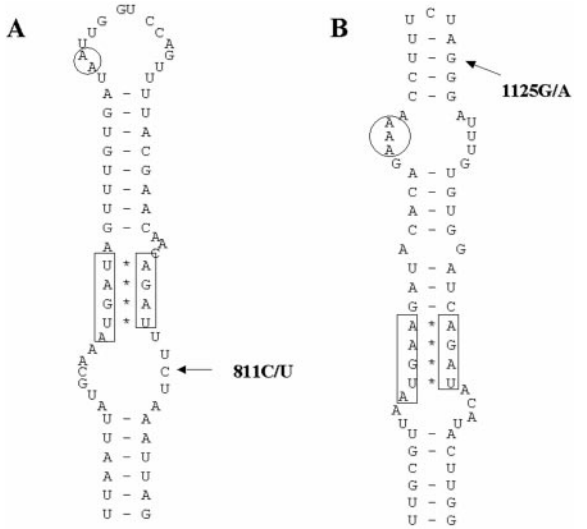

FIG. 3. **SECIS element structures in the 3′-UTR of the human 15-kDa selenoprotein gene.** *A*, the SECIS-like structure located at positions 754–819 is shown. This structure contains the C^{811}/T^{811} polymorphism in the internal loop and was found not to be functional. *B*, the sequence of the SECIS element located at positions 1090–1154 is shown. This structure contains the A^{1125}/G^{1125} polymorphism in the ministem of the apical loop and was found to be functional (see Figs. 4 and 5). The non-Watson-Crick Quartet sequence and a preceding A are *boxed*, and the conserved AA motif in the apical loop is *circled*.

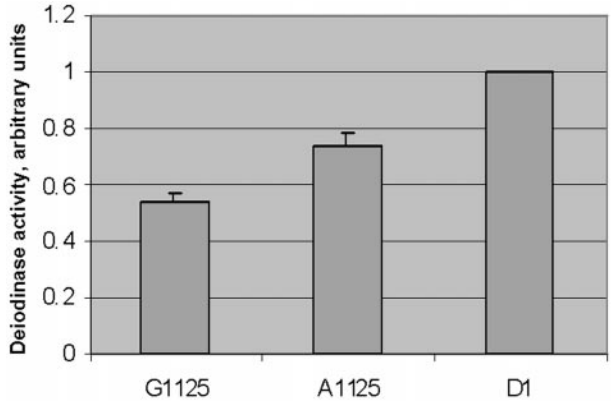

FIG. 4. **Incorporation of Sec into thyroid hormone deiodinase 1 with the 15-kDa protein SECIS element.** Constructs were prepared and assayed as described under "Experimental Procedures." G^{1125} and A^{1125} are two natural polymorphic forms of the 15-kDa protein SECIS element that were used to test Sec incorporation into thyroid hormone deiodinase. *D1* indicates a control assay that utilized the thyroid hormone deiodinase 1 natural SECIS element. The data shown are the result of three independent measurements.

dinase to about 75% of that observed in the wild type construct. Thus, G^{1125} seems to be a less active template for Sec insertion into a nascent selenopeptide when the growth medium was not supplemented with selenium (Fig. 4).

Further computer analysis of the human selenoprotein gene revealed the presence of an additional SECIS-like structure (Fig. 3). This structure satisfied criteria for both the primary sequence and secondary structure of the SECIS element, although the free energy for this structure was not as low as in most known human SECIS elements, and the structure could not be found with SECISearch (9). Nevertheless, this structure was thermodynamically similar to the SECIS elements in the human selenoprotein P gene, suggesting that it may potentially be a functional unit. Interestingly, the second polymorphic site found in the human selenoprotein gene, C^{811}/T^{811}, was located in this second SECIS-like structure (Fig. 3).

A possible role of the SECIS-like element and the C^{811}/T^{811} polymorphic site located in the internal loop of this structure in inserting Sec into a protein was tested by making a construct that contained this stem-loop structure in place of a wild-type SECIS element in the deiodinase gene. Transfection of either C^{811} or T^{811} form of this construct into HEK 293 cells revealed that the SECIS-like structure was not functional (data not shown). Thus, the human 15-kDa protein gene apparently has only one functional SECIS element.

Functional Consequences of the Polymorphic Sequences Located in the 15-kDa Protein SECIS Elements—Having determined that the G^{1125}/A^{1125} polymorphic site resided in the sequence capable of functioning as a SECIS element and that the identity of the 1125 nucleotide influenced the amount of deiodinase activity in the transfected cells, we next evaluated this phenomenon in greater detail. To quantitatively measure SECIS function, we utilized a reporter plasmid specifically designed to measure the ability of inserted sequences to promote the read-through of a UGA codon strategically placed between the genes for β-galactosidase and luciferase (30). Transient transfection of this plasmid resulted in detectable β-galactosidase in extracts prepared from transfected tissue culture cells. If a functional SECIS sequence was inserted in the polycloning region of the 3′-UTR, then translation of the UGA resulted in read-through into the luciferase gene and detectable activity in the prepared extracts. The ratio of luciferase to β-galactosidase activities was therefore a measure of SECIS efficiency of the inserted sequence normalized to the transfection efficiency as indicated by the β-galactosidase levels.

Segments of DNA spanning the 15-kDa protein SECIS sequence representing either the T^{811}/A^{1125} and C^{811}/G^{1125} haplotype were generated by PCR, cloned into the pBPLUGA reporter plasmid, and transfected along with the pSV_2neo into NIH 3T3 mouse fibroblasts. Extracts prepared from pBPLUGA-CG or pBpLUGA-TA plasmids yielded significant luciferase and β-galactosidase activities, while the parental pBPLUGA plasmid yielded only β-galactosidase activity. Consistent with the deiodinase assays (Fig. 4), the luciferase activity was higher for pBPLUGA-AT transfected cells compared with those containing pBpLUGA-CG (data not shown).[2] The tissue culture medium used for mammalian cells is generally regarded as being selenium-deficient as compared with human serum. We therefore determined whether there was a difference in SECIS function between the two plasmids as a function of increasing selenium concentration. As seen in Fig. 5, where the luciferase activity is normalized to base-line levels observed in unsupplemented medium, the reporter construct containing C^{811}/G^{1125} showed a greater response to added selenium than did that containing T^{811}/A^{1125}. These results collectively demonstrate that the identity of the nucleotides at 811/1125 influence the function of the 15-kDa protein SECIS element in a selenium-dependent manner.

cDNA for the Mouse 15-kDa Selenoprotein—Given the above described allelic variation in the 15-kDa gene in humans, we searched for similar genetic diversity in mice. We initially determined nucleotide sequences of mouse EST clones that were homologous to the human 15-kDa selenoprotein gene. Examination of these sequences indicated that the mouse cDNA sequence potentially encoded a protein of 162 residues. An N-terminal signal peptide spanning residues 1–27 of the mouse 15-kDa selenoprotein was predicted with sequence analysis programs. The mouse protein predicted by cDNA sequences was highly homologous to the human protein, and the mature proteins would differ by only 5 residues (Fig. 6). Likewise, the rat 15-kDa protein sequence, obtained by multiple sequence alignments of rat 15-kDa protein EST sequences,

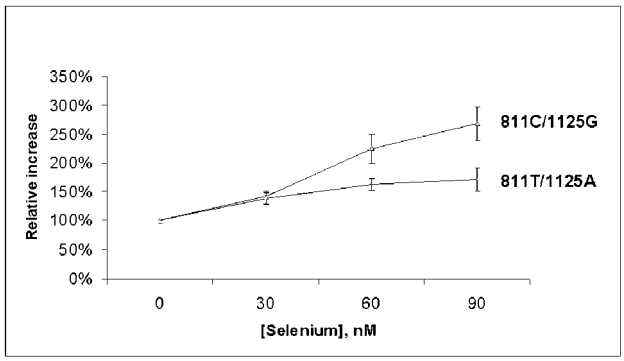

FIG. 5. **Effect of nucleotide identities at positions 811 and 1125 on SECIS function.** NIH 3T3 cells transfected with either pBPLUGA-CG (△) or pBpLUGA-TA plasmids (30), containing the polymorphic sequences C^{811}/G^{1125} and T^{811}/A^{1125}, respectively, were cultured for 5 days in the indicated amount of selenium, after which cells were harvested and lysates were generated for the analysis of luciferase and β-galactosidase activities. The luciferase activity was normalized by dividing by the measured β-galactosidase activity, and the data presented in the *figure* represent the percentage increase over base-line luciferase/β-galactosidase obtained from cultures without added selenium. The data are the average of the analysis of at least three independent cultures, and the *error bars* represent the S.E.

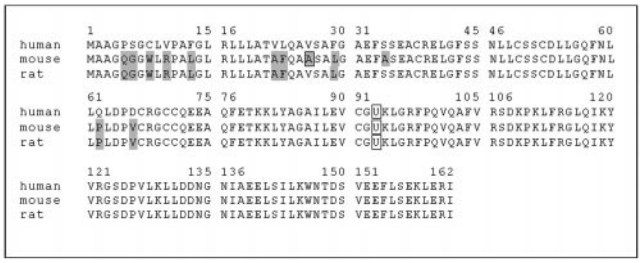

FIG. 6. **Homology among human, mouse, and rat 15-kDa proteins.** The human sequence, previously determined (Ref. 11; accession number NM004261), the mouse sequence determined herein, and the rat sequence derived from multiple alignments of rat EST sequences are compared. Mouse and rat residues that are different from the human 15-kDa protein sequence are *highlighted*. Sec residues (shown as *U*) in all three sequences and a variant amino acid residue 26 in the mouse sequence are shown as *boxes*.

differed from the human counterpart by 3 residues and the mouse by 2. The greatest conservation of gene sequence was present within the last 96 residues, which were identical in all three mammalian proteins, while all sequence variations occurred in the N-terminal portions of proteins.

The mouse 15-kDa protein gene was represented in dbEST by more than 100 sequences. As was observed in the human gene, analysis of these sequences suggested the presence of two major polymorphic forms corresponding to two alleles. These differed by at least 5 nucleotides (form 1: C^{323}, T^{438}, G^{681}, A^{1166}, and T^{1275}; form 2: T^{323}, C^{438}, A^{681}, G^{1166}, and G^{1275}). The first three nucleotide variations were located in the coding region, and the last two were in the 3′-UTR. Polymorphisms at nucleotides 438 and 681 did not result in changes in selenoprotein amino acid sequence, while substitution of nucleotide 323 resulted in a change from alanine to valine at position 26 in the protein sequence. None of the polymorphic positions were located in a single predicted SECIS element identified in the mouse sequence, and the closest polymorphic site to that element was located 10 nucleotides upstream. Form 1 was 4 times more abundant than form 2 among mouse EST sequences, and it was present in at least eight different mouse strains. Form 2 was present exclusively in the Barstead colon library (strain FVB/N) and in the Soares mammary gland NbMMG library (strain C57BL/6J). Further analysis of these two strains revealed that previously sequenced ESTs generated from the FVB/N strain included only form 2, while those sequences reported from the C57BL/6J strain contained both forms of the selenoprotein gene.

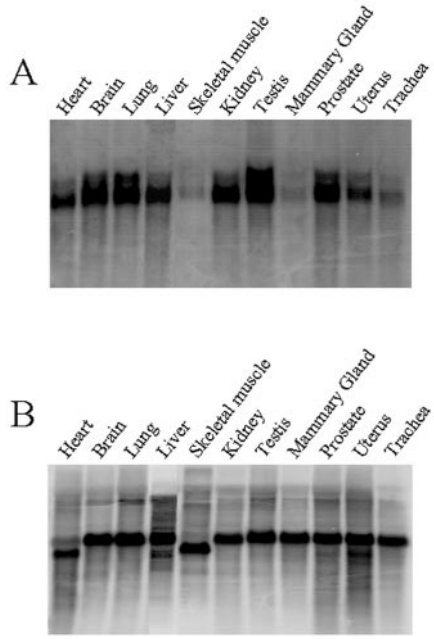

FIG. 7. **Northern blot analysis of human 15-kDa protein mRNA.** Expression of the 15-kDa protein mRNA was examined by Northern blot analysis in various tissues as shown. The blot was hybridized with the human cDNA probe (shown in *A*), stripped, and then hybridized with β-actin probe (shown in *B*).

Expression and Translation of 15-kDa Protein mRNAs—Northern blot analysis of the human 15-kDa protein mRNA is shown in Fig. 7. The 1.3-kb human selenoprotein mRNA was expressed in high levels in prostate, liver, brain, kidney, and testis, while it was essentially undetectable in muscle, mammary gland, and trachea. Several human tissues also exhibited an additional 15-kDa protein mRNA band of 1.6 kb that was present in minor levels in tissues such as lung, prostate, and uterus but present in major levels in testis. This band corresponds to an extended human 15-kDa protein cDNA sequence (containing a 275-nucleotide extension of exon 5) as observed in a portion of human EST sequences (see above).

The levels of expression of the mouse 15-kDa protein mRNA were assessed by Northern blot analysis as shown in Fig. 8 and corresponded to the expression pattern observed for the human 15-kDa protein mRNA (Fig. 8). As expected, the size of the mouse 15-kDa protein mRNA was ~1.4 kb in all tissues examined except testis, where the analysis indicated two hybridizing bands, one of ~1.4 kb and another of ~1.7 kb (Fig. 8).

To assess the contribution of the 1.4- and 1.7-kb mRNAs to 15-kDa protein synthesis, a blot prepared with mRNA isolated from polysomal and nonpolysomal fractions from mouse testis was hybridized with the appropriate cDNA probe. Interestingly, the nonpolysomal fraction contained approximately equal amounts of both mRNA forms, while the polysomal fraction contained greater than 90% of the 1.4-kb mRNA (data not shown).

Expression of the Mouse 15-kDa Selenoprotein—Levels of the 15-kDa protein were examined in several mouse tissues and cells by Western blot analyses. Isolated native human protein was used as a standard to determine the size of the 15-kDa protein detected with our antibodies in mammalian cells. The data suggest that the major form of the protein in mouse cells was the form lacking the N-terminal signal peptide, since the

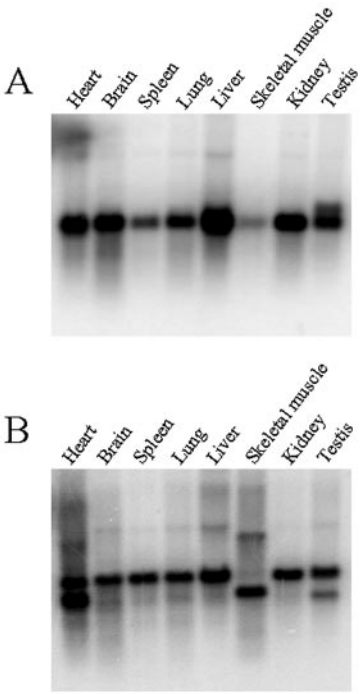

FIG. 8. **Northern blot analysis of mouse 15-kDa protein mRNA.** Expression of the 15-kDa protein mRNA was examined by Northern blot analysis in various tissues as shown. The blot was hybridized with the mouse cDNA probe (shown in *A*), stripped, and then hybridized with β-actin probe (shown in *B*).

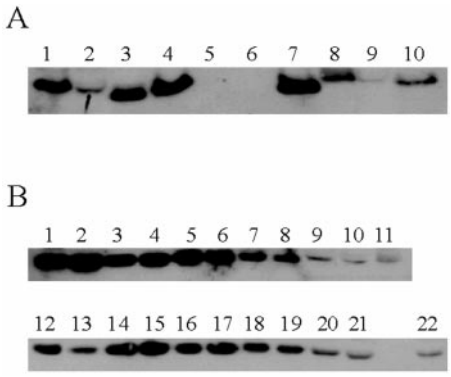

FIG. 9. **Immunoblot detection of the mouse 15-kDa selenoprotein in tumors and normal tissues.** Expression of the 15-kDa protein was examined in various normal and malignant cells and tissues by Western analysis as shown. *A*, *lane 1*, liver of TGFα/*c-myc* transgenic mouse (10 months); *lane 2*, liver tumor of TGFα/*c-myc* mouse (from the mouse as in *lane 1*); *lanes 3* and *7*, mouse prostate (wild type); *lanes 4* and *10*, purified 15-kDa selenoprotein from human T cell line JPX9; *lanes 5* and *6*, mouse prostate adenocarcinoma cell line Pr-14 (20); *lane 8*, liver of TGFα/*c-myc* mouse (10 months); *lane 9*, tumor liver of TGFα/*c-myc* mouse (from the same mouse as in *lane 8*). *B*, *lanes 1* and *2*, liver of wild type mice (2.5 months); *lanes 3* and *4*, liver of TGFα/*c-myc* mouse (2.5 months); *lanes 5* and *6*, liver of TGFα/*c-myc* mouse (2.5 months); *lanes 7* and *8*, liver of TGFα/*c-myc* mouse (10 months); *lanes 9–11*, liver tumors of TGFα/*c-myc* mouse (10 months); *lanes 12* and *13*, liver of wild type mouse (1 month); *lanes 14* and *15*, liver of TGFα/*c-myc* mouse (1 month); *lanes 16* and *17*, liver of TGFα/*c-myc* mouse (1 month); *lanes 18* and *19*, liver of TGFα/*c-myc* mouse (10 months); *lanes 20–22*, liver tumors of TGFα/*c-myc* mouse (10 months). All samples were obtained from different mice. Equal protein amounts were loaded on each *lane*.

detected mouse protein migrated similarly to the 15-kDa protein standards that lacked the N-terminal sequence.

Altered expression of the 15-kDa protein may be significant in the etiology of tumor development and/or the mechanism by which selenium functions in cancer prevention. The above described Western analysis indicated relatively high levels of expression in both the prostate and liver, two organs previously indicated as being responsive to the chemopreventive effects of selenium (13–15). Previously, we have examined selenoprotein levels in transgenic TGFα/c-*myc* mice (22), which is a mouse model for accelerated hepatocarcinogenesis (21). In that study (22), it was found that GPx1 levels were significantly decreased in liver tumors compared with surrounding liver tissue, whereas levels of another major selenoprotein, TR1, were slightly elevated. Using this model, levels of the 15-kDa selenoprotein were assessed in liver tumors and adjacent tissue by Western blotting. Two pairs of matched samples (from the same animals) and individual tumor and normal livers (Fig. 9*B*) were analyzed, and in each case the levels of the 15-kDa protein were lower in the tumors. In addition to the observed difference in expression of the selenoprotein in hepatocarcinomas, the 15-kDa protein was not detectable in mouse prostate adenocarcinoma cells, while normal mouse prostate showed a strong signal with 15-kDa protein-specific antibodies (Fig. 9*A*).

DISCUSSION

The 15-kDa selenoprotein is among the more recently discovered proteins shown to contain the Sec amino acid encoded by an in-frame UGA codon (11). Previously, only the cDNA sequence obtained from characterization of a human EST clone was available. In this work, the organization and sequence of the gene for this protein were determined from human genomic DNA. The human gene spans 51 kb and is organized in five exons and four introns with the Sec-encoding TGA codon in exon 3 and a SECIS element in exon 5. The human 15-kDa selenoprotein gene was located on chromosome 1 at position p31. This region is commonly mutated or deleted in human cancers, and the presence of a tumor suppressor gene on 1p31 has been suggested (18, 19). The loss of one copy of the 15-kDa protein gene may result in the decrease in selenoprotein expression in malignant tissues relative to the corresponding normal tissue.

Analyses of publicly available cDNA sequences derived from the 15-kDa protein gene revealed two single nucleotide polymorphisms at positions 811 and 1125 in the human cDNA. These were organized into two alleles, C^{811}/G^{1125} and T^{811}/A^{1125} in the 68%/32% frequency distribution. Only these two haplotypes were observed in cDNA sequences submitted to date and this is also the case in over 700 human DNA samples examined.[2]

The human 15-kDa protein gene gives rise to several mRNA forms. The major mRNA form has been previously described (11), and we now detected additional human mRNA forms that lack exon 4 or contain extended exon 5. We found that exon 1 encodes an N-terminal signal peptide spanning amino acid residues 1–27 of the human sequence that is also present in rodent sequences, suggesting that the mammalian 15-kDa protein is a compartmentalized protein. This conclusion is supported by the observation that N-terminal amino acid residues are not present in the isolated human 15-kDa protein (11).

Previously, the presence of the SECIS element in the 3′-UTR of the human 15-kDa protein gene was predicted by structural similarity to other selenoprotein genes. In this work, we established that this element was indeed functional by demonstrating that it could replace the natural SECIS element of the thyroid hormone deiodinase 1 gene with comparable efficiency. Furthermore, these sequences were able to support the readthrough of an in-frame UGA codon separating the β-galactosidase and luciferase genes in a reporter construct specifically designed for this purpose.

Since the 1125 polymorphic site was located in the apical loop of a functional SECIS element, we tested the hypothesis that the identity of the nucleotide at this position may influ-

ence Sec incorporation. We initially found that A^{1125} was more efficient than G^{1125} in supporting synthesis of active thyroid hormone deiodinase. In this experiment, the growth medium was supplemented with 10% fetal calf serum, which may be considered as a modest selenium deficiency, since serum is the major source of selenium for protein synthesis under cell culture conditions and the level of selenium in 10% fetal calf serum-supplemented medium is low (29). We further extended these observations by employing a β-galactosidase-UGA codon-luciferase-SECIS construct, in which the sequence spanning both polymorphisms in the human 15-kDa selenoprotein gene was included in the construct. Using transiently transfected cells, we were able to detect differences in SECIS element function between the two naturally occurring alleles (C^{811}/G^{1125} and T^{811}/A^{1125}) of the 15-kDa protein gene. The SECIS elements from these two haplotypes differed in their activity in medium unsupplemented with selenium and the same medium supplemented with 30–90 nM sodium selenite (Fig. 5). This finding was also supported by the analysis of stably transfected cells and nonnatural polymorphic forms of the 15-kDa protein gene.[2]

Thus, natural polymorphisms found in the 3′-UTR of the 15-kDa protein gene influence expression levels of the selenoprotein gene product, and this occurs in a selenium-dependent manner. It is therefore possible that individuals representing different combinations of these two haplotypes may express different amounts of the 15-kDa selenoprotein and, in addition, they may differentially respond to changes in dietary selenium (*i.e.* differentially control expression levels of the 15-kDa protein in response to changes in selenium levels). The role of natural polymorphisms in affecting Sec incorporation into a selenoprotein and influencing selenoprotein levels as well as the role of selenium in these processes have not been previously described for any other selenoprotein.

In addition to the human selenoprotein gene, the existence of two haplotypes was detected in the mouse 15-kDa protein cDNA sequences. However, in the mouse, multiple sequence differences between the two alleles were detected. At present the functional consequences of these polymorphisms remain unknown. We also detected two forms of the mouse 15-kDa protein mRNA, but in contrast to a human gene, the longer form of mouse mRNA was only expressed in testes. Although both forms were present in equal amounts in mouse testes, the shorter one was predominantly associated with polysomes, suggesting that it is utilized in protein synthesis to a far greater extent than the longer form. Examination of the expression pattern of the 15-kDa protein indicated that various tissues expressed very different levels of both this protein and its mRNA.

It is noteworthy that prostate and liver in both humans and mice express relatively high levels of the 15-kDa protein. Human selenium supplementation trials have suggested that dietary selenium can reduce cancer incidence in these two organs (15), and it is likely that the most protection is provided to those with lower selenium intake. Furthermore, epidemiological data have indicated a statistically significant inverse correlation between selenium in the diet and prostate cancer (13). However, the mechanism of cancer prevention by selenium is poorly characterized, and no selenoprotein has been implicated in such protection. The data presented in this study raise the possibility that the 15-kDa protein may function in the prevention of cancer and possibly serve as an agent by which selenium supplementation exerts its chemopreventive effect. Consistent with this notion is our observation that liver tumors expressed reduced levels of the 15-kDa protein compared with adjacent hepatic tissue in the TGFα/c-*myc* mouse model used in this study. In addition, 15-kDa selenoprotein levels were essentially undetectable in a mouse prostate cancer cell line, while this protein was abundant in a normal mouse prostate. If lower levels of the 15-kDa protein predispose to malignant transformation, then the observation that the difference in SECIS element function between the two naturally occurring alleles in the human population may indicate a segment of the population who are either at greater risk of cancer or might benefit from selenium supplementation. The above observations and the fact that the region of chromosomal location of the 15-kDa protein gene is often associated, following deletion or mutation, with malignancy suggest a possible role of the 15-kDa protein in cancer etiology.

Acknowledgments—We thank Drs. V. Factor, L. Flohé, C. Jorcyk, and J. Green for providing reagents and Dr. V. M. Chennathukuzhi for advice and assistance in preparing blots containing mRNA isolated from polysomes.

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